

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
22 January 2004 (22.01.2004)

PCT

(10) International Publication Number
WO 2004/007757 A1

(51) International Patent Classification⁷:
1/68, 1/70, G01N 33/53, 33/566, 33/567

C12Q 1/00,

(74) Agents: KILYK, John, Jr. et al.; Leydig, Voit & Mayer,
Ltd., Suite 4900, Two Prudential Plaza, 180 North Stetson
Ave., Chicago, IL 60601-6780 (US).

(21) International Application Number:

PCT/US2003/019584

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,
SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG,
UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date: 20 June 2003 (20.06.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/395,038

11 July 2002 (11.07.2002) US

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant: THE AMERICAN NATIONAL RED
CROSS [US/US]; 8111 Gatehouse Road, Falls Church,
VA 22042 (US).

(72) Inventors: HAMMOND, David, J.; 4916 Ripplemead
Ct., Laytonsville, MD 20882 (US). LATHROP, Julia,
Tait; 100 Poplar Drive, Falls Church, VA 22046 (US).
SARKAR, Jolly; 2559 Little Vista Terrace, Olney, MD
20832 (US). GHEORGHIU, Liliana; 4400 East West
Hwy, Apt. 517, Bethesda, MD 20814 (US).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR IDENTIFYING INDIVIDUAL ACTIVE ENTITIES FROM COMPLEX MIXTURES

(57) Abstract: A method of screening a mixture for active entities, which method comprises: providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes, contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligandsupport complex, separating at least one entity-ligand-support complex from the unbound entities, assaying at least one entity of at least one entity-ligandsupport- complex for an activity, detecting the activity, and selecting at least one entity-ligand-support-complex having the entity, which exhibited the detected activity, whereupon a mixture is screened for active entities; and related methods.

WO 2004/007757 A1

METHOD FOR IDENTIFYING INDIVIDUAL ACTIVE ENTITIES FROM COMPLEX MIXTURES

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 60/395,038, filed July 11, 2002.

FIELD OF THE INVENTION

[0002] This invention pertains to methods of screening a mixture for active entities.

BACKGROUND OF THE INVENTION

[0003] Conventional proteomics seeks to generate a comprehensive identity profile of the entire proteome of an organism and, through analysis of this information, to identify potential diagnostic and therapeutic entities. Currently, the dominant technologies for resolving protein mixtures are two-dimensional gel electrophoresis and multi-dimensional liquid chromatography, both coupled to mass spectrometry. An example of the power of this approach is the resolution and identification of 1,484 proteins in yeast (Washburn et al., *Nat. Biotechnol.*, 9(3): 242-2471 (2001)). A quantitative methodology for protein separation and identification is isotope coded affinity tag (ICAT), developed by Aebersold and colleagues (Smolka et al., *Anal. Biochem.*, 297(1): 25-312 (2001)). ICAT involves the site-specific, covalent labeling of proteins with isotopically normal or heavy reagents to quantitate levels of protein expression. Yet another example of methodology that separates and identifies proteins is a global version of the yeast two-hybrid screening assay developed by Uetz et al. (Uetz et al., *Nature*, 403(6770): 623-627 (2000)) and Ito et al. (Ito et al., *Proc. Natl. Acad. Sci. USA*, 98(8): 4569-4574 (2001)), which identified over 4,000 protein-protein interactions in *Saccharomyces cerevisiae*. Although these approaches for separating and identifying proteins are powerful, they do not identify the cellular functions of the fractionated proteins.

[0004] Complex protein mixtures also have been separated on libraries of combinatorially-generated ligands immobilized on beads. Combinatorial and synthetic chemistry techniques well-known in the art can generate libraries of millions of ligands, (Lam et al., *Nature*, 354: 82-84 (1991) and International (PCT) Patent Application WO 92/00091) each of which may have the capacity to bind to molecules. A library of linear hexamer ligands made with 18 of the natural amino acids, for example, contains 34×10^6 different structures. When amino acid analogs and isomers are also included, the number of potential structures is practically limitless. Each bead essentially has millions of copies of a

single structure on its surface and different beads contain different sequences. Moreover, the total number of beads in a library may be enormous.

[0005] Following exposure of an entity molecule to a combinatorial library, the entity will bind through affinity interactions to specific ligands within the library. At equilibrium, the concentration of the entity on the support, *e.g.*, a bead bearing an affinity ligand, will be dependent on the affinity constant and the concentration of the ligand and the entity. In published screening methodologies, the detection of the bound entity and hence its ligand can be straightforward when a purified, radiolabeled initial entity is used (Mondorf et al., *J. Peptide Research*, 52: 526-536 (1998)). Other methods include detection by an antibody against the entity (Buettner et al., *International Journal of Peptide & Protein Research*, 47: 70-83 (1996); Furka et al., *International Journal Peptide Protein Research*, 37(6): 487-493 (1991); and Lam et al., (1991) *supra*). Ligands to multiple entities can be detected using beads immobilized on an adhesive in combination with a subtractive screening method. This is referred to as the QuASAR method (International (PCT) Patent Application WO 01/40265) and was used to detect ligands that bound to virus and prion protein.

[0006] In a related but distinct methodology using a bead-based library, the entity protein in its normal physiological environment is incubated with a peptide ligand library bound to beads. Following fractionation, the beads are immobilized and arrayed in a thin gel of low melting-point agarose to create a crude "array" of ligands. A protein-binding membrane (nitrocellulose or PVDF) is laid on the gel, and the proteins are eluted from the beads under a variety of conditions and captured in the same relative position as they were in the gel on the membrane by unidirectional capillary transfer of solvent diffusing through the gel (similar to a Southern transfer of DNA). As all of the proteins that were present in the original mixture that bound to ligands immobilized on beads are present on the membranes, the membranes themselves can be stripped and reprobed for different, known proteins.

[0007] In the foregoing methods, in which a library of combinatorially-generated ligands immobilized onto beads is used, entity proteins are simply separated and identified based on their binding to the ligands and not on their biochemical or biological function. Therefore, there exists a need in the art for methods that separates and identify proteins from complex mixtures based on their chemical, physical, biological, and/or biochemical function and not merely on their ability to bind a ligand within the library.

[0008] The invention provides such methods. This and other objects and advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

BRIEF SUMMARY OF THE INVENTION

[0009] The invention provides methods of screening a mixture for active entities. In a first method, the method comprises (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes, (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex, (iii) separating at least one entity-ligand-support complex from the unbound entities, (iv) assaying at least one entity of at least one separated entity-ligand-support complex for an activity, (v) detecting the activity, and (vi) selecting at least one entity-ligand-support-complex having the entity, which exhibited the detected activity.

[0010] In a second method, the method comprises (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes, (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex, (iii) separating at least one entity-ligand-support complex from the unbound entities, (iv) separating at least one entity-ligand-support complex and the ligand-support complexes into pools, (v) dissociating at least one entity from at least one separated entity-ligand-support complex, (vi) removing from the pools the ligand-support complexes or the at least one dissociated entity of step (v), (vii) assaying at least one dissociated entity of step (v) for an activity, (viii) detecting the activity, and (ix) selecting at least one entity exhibiting the detected activity.

[0011] In a third method, the method comprises (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes, (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex, (iii) separating at least one entity-ligand-support complex from the unbound entities, (iv) separating at least one entity-ligand-support complex and the ligand-support complexes into pools, (v) adding a semi-solid or viscous material to each pool, wherein the entity of at least one separated entity-ligand-support complex dissociates from the complex and diffuses into the material, thereby forming a concentration gradient of the entity, wherein the concentration of the entity gradually decreases as the distance from the ligand-support complex from which at entity dissociated increases, (vi) assaying at least one dissociated entity of step (v) for an activity, (vii) detecting the activity, and (viii) selecting the at least one entity exhibiting the detected activity.

DETAILED DESCRIPTION OF THE INVENTION

[0012] The invention provides methods of screening a mixture for active entities. In a first method, the method comprises (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes, (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex, (iii) separating the at least one entity-ligand-support complex from the unbound entities, (iv) assaying the at least one entity of the at least one entity-ligand-support complex for an activity, (v) detecting the activity, and (vi) selecting the at least one entity-ligand-support-complex having the entity, which exhibited the detected activity.

[0013] The term "mixture" as used herein refers to any collection comprising more than one entity, wherein the term "entity" as used herein refers to any biological, chemical, or biochemical entity or target, such as a compound, molecule, virus, or cell. Preferably, the mixture is a collection of different entities, each having a different chemical identity, e.g., molecular formula, chemical structure, nucleotide sequence or amino acid sequence, or each having a different physical identity, e.g., spectral signal or conformation. More preferably, the mixture is a collection comprising a plurality of different entities. One of ordinary skill in the art appreciates that the mixture can comprise one or more copies of each entity having a different chemical or physical identity.

[0014] The entities comprising the mixture can be isolated from nature or synthetically produced, and can be organic or inorganic in nature (e.g., a synthetic inorganic compound or a synthetic organic compound). For example, the entity can be a drug or drug candidate (such as a small molecule drug candidate), a fertilizer component, an insecticide component, or a derivative, analogue, or enantiomer thereof. In addition, the entity can be endogenous or exogenous to any prokaryote or eukaryote, e.g., a bacterium, a fungus, yeast, a plant, or a mammal. Suitable entities for the inventive method include, but are not limited to, cells (e.g., stem cells or cells in culture), proteins, peptides, drugs, antibodies, synthetic molecules, organic compounds, protein complexes (e.g., blood clotting Factor XIII and fibrinogen or blood clotting Factor VIII and Von Willebrand Factor), bacteria, viruses, fungi, yeast, prions, amino acids, nucleic acids, carbohydrates, lipids, isoforms of any of the foregoing, and combinations of any of the foregoing. Preferably, the entities are proteins. Suitable protein entities include, for example, receptors, antibodies, immunogens, enzymes (e.g., proteases), and enzyme substrates. More preferably, the proteins are plasma-derived proteins. Most preferably, the plasma-derived proteins are immunoglobulins, e.g., IgG,

IgM, IgA, IgE. The immunoglobulins can be from an organism in a diseased state, (and optionally not found in the plasma of a healthy subject) or produced as a result of the administration of an agent, e.g., a drug. Alternatively, it is preferred that the entities are cells. More preferably, the cells are stem cells.

[0015] The entity of the inventive methods can be obtained from any source, i.e., the mixture comprising the entities can be any complex mixture, such as extracts of soil, air, water, food, swabs for evaluating environmental contamination, intermediate or end-stage chemical reaction mixtures, and the like. The mixture comprising the entity can be a chemical or synthetic mixture and can be present in a combinatorial library and/or present in organic solvents under extreme conditions of pressure, temperature, etc. Preferably, the mixture is a biological fluid, an environmental extract, or a composition comprising chemical compounds.

[0016] By "biological fluid" is meant any aqueous solution that is derived from a prokaryotic or eukaryotic organism. The biological fluid can be obtained directly from the prokaryotic or eukaryotic organism, such as blood, lymph, tears, saliva, perspiration, and urine. Alternatively, the biological fluid can be obtained by culturing cells of the organism, such as fermentation broth and cell culture medium. Suitable biological fluids for use in the inventive method include, but are not limited to, blood, plasma, pooled plasma, intermediates of plasma fractionation, serum, a cell homogenate, a tissue homogenate, a conditioned medium, a fermentation broth, cerebrospinal fluid, urine, saliva, milk, ductal fluid, tears, perspiration, lymph, semen, umbilical cord fluid, and amniotic fluid. Preferably, the biological fluid is a plasma-derived fraction comprising antibodies and anti-idiotypic antibodies. By "anti-idiotypic" as used herein refers to an antibody that has an epitope that is specific for the antigen-determining region of another antibody. Also preferred is that the biological fluid is obtained from a host afflicted with a disease. The term "host" as used herein refers to any eukaryotic or prokaryotic organism, e.g., bacteria, virus, yeast, fungi, bird, reptile, and mammal. The disease, which afflicts the host, can be any disease including any condition, malady, infection, and the like. For instance, the disease can be cancer, diabetes, an autoimmune disease, osteoporosis, wound healing, liver regeneration, or lung disease. Alternatively, the disease can be an infection with a parasite, virus, or bacteria.

[0017] The term "environmental extract" as used herein refers to any sample taken from an environment. The environment can be a natural environment, such as a naturally-occurring body of water. Alternatively, the environment can be a man-made environment, such as a building. In this respect, the environmental extract can be a soil, soil extract, an extract from a naturally-occurring body of water, a sample of ice, air, ash, rock, or

permafrost, or a swab from a building. The naturally-occurring body of water can be, for example, an ocean, a lake, a sea, a river, a swamp, a pond, a delta, or a bay. The environmental extract can alternatively be an extract from a water treatment center. The building can be any man-made building. Preferably, the building is contaminated with one or more toxic agents, such as sarin, soman, nerve poisons, explosive chemicals, pesticides, pathogens, VX, and blister agents.

[0018] The composition comprising chemical compounds can comprise natural or synthetic chemical compounds. For instance, the composition can be a chemical or synthetic mixture of reaction products. Alternatively, the composition can be present in a combinatorial library and/or present in organic solvents under extreme conditions of pressure, temperature, etc.

[0019] The term "ligand" as used herein refers to any biological, chemical, or biochemical agent, such as a compound, molecule, or cell that binds to an entity. The ligand can be isolated from natural or synthetically produced materials. The ligand can be endogenous or exogenous to a prokaryote or eukaryote, e.g. bacteria, a fungus, yeast, plant, or a mammal. Suitable ligands for the inventive methods include, but are not limited to, cells, bacteria, viruses, yeast, proteins, peptides, amino acids, nucleic acids, carbohydrates, lipids, drugs, synthetic inorganic compounds, synthetic organic compounds, antibody preparations (e.g., antibody fragments, chemically-modified antibodies, and the like), sugars, isoforms of any of the foregoing, and combinations of any of the foregoing.

[0020] Organic molecules include, for example, synthetic organic compounds typically employed as pharmacotherapeutic agents. Such molecules are, optionally, mass-produced by combinatorial synthetic methods or, more specifically, by strategic syntheses devised to arrive at specific molecules. Likewise, organic molecules also include natural products and analogues, whether extracted from their natural environment or strategically synthesized. The term "organic" as used herein is not intended to be limited to molecules comprised only of carbon and hydrogen, but rather is used in its broader sense as encompassing macromolecules of biological origin.

[0021] Preferably, the ligands are peptides. The term "peptide" as used herein refers to an entity comprising at least one peptide bond, and can comprise either D and/or L amino acids. Ideally, the ligand is a peptide consisting essentially of about 2 to about 10 amino acids (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids). Desirably, the peptide ligands are generated by combinatorial approaches, i.e., techniques commonly employed in the generation of a combinatorial library, e.g., the split, couple, recombine method or other approaches known in the art (see, e.g., Furka et al., *Int. J. Peptide Protein Res.*, 37: 487-493 (1991); Lam et al., *Nature*, 354: 82-84 (1991); International Patent Application WO

92/00091; and U.S. Patents 5,010,175, 5,133,866, and 5,498, 538). The expression of peptide libraries also is described in Devlin et al., *Science*, 249: 404-406 (1990). In peptide libraries, the number of discrete peptides of differing sequence increases dramatically with the number of coupling reactions performed, the size of the peptide, and the number of distinct amino acids utilized. For example, the random incorporation of 19 amino acids into pentapeptides produces up to 2,476,099 (19^5) individual peptides of differing sequence (Lam et al., *supra*). Combinatorial methods allow generation of libraries of ligands directly on a support. Typically, the ligands are synthesized on particles of support media such that multiple copies of a single ligand are synthesized on each particle (e.g., bead), although this is not required in the context of the invention.

[0022] In the inventive methods, each of the ligands is attached to a support, thereby achieving formation of ligand-support complexes. The term "support" as used herein refers to any support matrix, such as those solid supports known in the art, which serve to immobilize the ligand. Suitable supports include, but are not limited to, membranes, filters, meshes, or particles comprising cellulose, acrylics, polyacrylamide or polyhydroxylated methacrylate polymers, polystyrene, dextran, agarose, polysaccharides, hydrophilic vinyl polymers, polymerized derivatives of any of the foregoing, and combinations of any of the foregoing, as well as any porous or non-porous matrix to which ligands can be directly attached or on which ligands can be synthesized. Preferably, the support is inert such that chemical reaction with the entity and/or the immobilized ligand is minimized. In this regard, the support desirably comprises a polymethacrylate, polyacrylate, agarose, a polyacrylamide, dextran, cellulose, a polysaccharide, nitrocellulose, silicon, styrene, metal, polyethylene-coated polystyrene, polyvinylidene fluoride, nylon, or a combination of any of the foregoing. Particularly preferred support materials are polyethylene coated polystyrene and polymethacrylate. Various resins are commercially available, and, preferably, the support is a resin bead, such as a chromatographic resin bead.

[0023] Many solid supports displaying potential ligands are commercially available. Alternatively, the ligands of the inventive method can be indirectly attached or directly immobilized on the support using standard methods (see, for example, Harlow and Lane, *Antibodies*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988); Biancali et al., *Letters in Peptide Science* 7(291): 297(2000); MacBeath et al., *Science* 289: 1760-1763 (2000); Cass et al., ed., *Proceedings of the Thirteenth American Peptide Symposium*; Leiden, Escom, 975-979 (1994); U.S. Patent 5,576,220; Cook et al., *Tetrahedron Letters* 35: 6777-6780 (1994); and Fodor et al., *Science* 251(4995): 767-773 (1991)). In one embodiment, the ligand(s) are synthesized on the surface of the support, which is advantageous in generating peptide libraries. The ligand(s) can be chemically conjugated to

the support or can be attached via linkers, beta alanine, glycine, polymers containing glycine-serine, short chain hydrocarbons of the formula $-(CH_2)-$, polyethylene glycol, epsilon amino caproic acid, and linkers comprising $-O(CH_2)_n$, wherein n is 1-30. If desired, the ligand(s) can be attached by one or by several different cleavable linkers, e.g., photolabile or acid labile moieties, enabling the selective detachment of a population of ligands for analysis. Ligands can be used, for example, as affinity purification media for proteins and enantiomeric separation (e.g., to concentrate, isolate, detect, characterize, quantify, or identify entities in a sample), as diagnostic therapeutic tools, catalysts and enhancers of chemical reactions, and as selective stabilizers of proteins.

[0024] Once formed, the ligand-support complexes are contacted with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex. Such conditions, as recognized by one of ordinary skill in the art, depend upon the mixture (entities) and the ligands themselves, in addition to other factors, such as pH, temperature, contact time, salt concentration, and the like. Determination of suitable conditions that allow at least one entity to bind to at least one ligand-support complex is within the ordinary skill. In a preferred embodiment of the invention, multiple entity-ligand support complexes are formed.

[0025] After at least one entity-ligand-support complex is formed, at least one entity-ligand-support complex and the ligand-support complexes are separated from the unbound entities. The term "unbound entities" as used herein refers to the entities of the mixture that do not bind or are loosely bound to any of the ligand-support complexes. Means of separating at least one entity-ligand-support complex from the unbound entities are known in the art and, include, for instance, centrifugation, serial dilution, filtration, dialysis, and washing in a chromatographic format.

[0026] The inventive methods can optionally comprise a sub-pooling step, wherein at least one entity-ligand-support complex and the ligand-support complexes are separated into several pools or sub-populations. Preferably, the sub-pooling step achieves on average about 10-500 entity-ligand-support complexes and ligand-support complexes per pool. More preferably, the average number of complexes per pool is 20-100. Most desirably, the complexes are sub-pooled, such that 50 complexes are present in each pool.

[0027] Furthermore, the inventive methods can optionally comprise, after the sub-pooling step, an eluting step, wherein the entities of the multiple entity-ligand-support complexes are dissociated from the complexes, and the ligand-support complexes are subsequently removed from the pools.

[0028] In this regard, the invention provides a second method of screening a mixture for active entities. This method comprises (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes, (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex, (iii) separating at least one entity-ligand-support complex and the ligand-support complexes from the unbound entities, (iv) separating at least one entity-ligand-support complex and the ligand-support complexes into pools, (v) dissociating at least one entity from at least one separated entity-ligand-support complex, (vi) removing from the pools the ligand-support complexes or the at least one dissociated entity of step (v), (vii) assaying at least one dissociated entity of step (v) for an activity, (viii) detecting the activity, and (ix) selecting at least one entity exhibiting the detected activity.

[0029] One of the advantages of this inventive method is that the entity being tested for activity dissociates from the ligand-support complex and is, thus, in an unbound state. It is therefore, uninhibited by the ligand and/or support and is free to interact with and act on the enzyme substrate or cells used in the activity assay. One of ordinary skill in the art recognizes that the degree of dissociation of the entity from the ligand-support complex need not be 100% or a complete dissociation, as it is possible that an activity of the entity can be detected with only some of the entity dissociated from the ligand-support complex.

[0030] Alternatively, the method could optionally further comprise, after the sub-pooling step, a step, wherein a semi-solid or viscous material is added to each pool, wherein the entity of at least one entity-ligand-support complex dissociates from the complex and diffuses into the material, thereby forming a concentration gradient of the entity, wherein the concentration of the entity gradually decreases as the distance from the ligand-support complex from which the entity dissociates increases. Suitable semi-solid materials for use in this method include, for instance, agarose, gelatin, glycerol, polyethylene glycol, acrylamide, and fibrin sealant. Preferably, the semi-solid material is 0.5% w/v agarose.

[0031] In this respect, the invention provides a third method of screening a mixture for active entities. This method comprises (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes, (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex, (iii) separating at least one entity-ligand-support complex and the ligand-support complexes from the unbound entities, (iv) separating at least one entity-ligand-support complex and the ligand-support

complexes into pools, (v) adding a semi-solid or viscous material to each pool, wherein the entity of the at least one separated entity-ligand-support complex dissociates from the complex and diffuses into the material, thereby forming a concentration gradient of the entity, wherein the concentration of the entity gradually decreases as the distance from the ligand-support complex from which the entity dissociates increases, (vi) assaying at least one dissociated entity of step (v) for an activity, (vii) detecting the activity, and (viii) selecting at least one entity exhibiting the detected activity.

[0032] The advantages offered by this method also include the entity being in a free, unbound state, uninhibited by the ligand-support complex to interact with or act on an enzyme substrate or cells used in the activity assay. In addition, this method offers the advantage of being able to test the activity of the entity in a dose-dependent manner.

[0033] In the inventive methods, the mixture is screened for active entities by assaying at least one entity-ligand-support complex for an activity. The term "active entity" as used herein refers to any entity of the mixture that exhibits the assayed activity or a serendipitous result. In this regard, most entities of a given mixture have the potential of being an active entity, depending upon the particular activity being assayed in the method. For purposes herein, the active entity will also be active in the sense that the entity binds to a ligand-support complex. The assayed activity can be any biological, physical, chemical, or biochemical activity, provided that the activity results in a detectable signal, such as the enzymatic modification of a substrate. A chemical activity, e.g., an activity directly related to the chemical composition of the entity, can be employed. In other words, the entity can be identified by the presence of specific chemical subunits or moieties or chemical structures. Physical properties useful in detection methods include, for example, spectral signal, which can be determined via fluorescence or mass-spectrometry, respectively. The means of detection need not detect the activity of the entity alone, but can selectively identify activity or activities of more than one entity or of an entity complex, e.g., an entity complexed with other biological entities such as co-factors or enzymes.

[0034] Preferably, the activity can be an enzyme activity or inhibition of an enzyme activity. For example, the inventive method can comprise performing an enzyme activity assay to characterize an entity on the basis of biological activity. An enzyme substrate is applied to at least one entity-ligand-support complex under conditions which allow for enzymatic modification of the substrate by the entity to form a product. The product is then detected, thereby identifying the presence of the entity. Alternatively, the lack of product formation could be detected in order to identify active entities that inhibit a given enzyme activity. Enzyme activity assays are further described in, for example, Haugland, *supra*.

[0035] The activity alternatively can be an effect on a cell, a cell population, a tissue, or a whole organism. In any of these instances, a cell-based assay is performed wherein at least one entity-ligand-support complex is contacted with cells on which the entity exerts some observable biologic effect. Purely by way of illustration, the preferred entity can be an antibacterial agent. The ligand of the inventive methods binds potential active sites of the antibacterial agent, thereby separating the antibacterial entity from a mixture (e.g., a library of potential therapeutics). A lawn of bacteria is applied to the entity, and the antibacterial entity is detected by zones of bactericidal activity. When the activity is an effect on a cell or a cell population, the effect can be, for instance, cell migration, cell proliferation, cell death, cell differentiation, cell cycle entry, cell cycle arrest, apoptosis, cell lysis, growth arrest, cell survival, a change in an intracellular signaling pathway, antigen expression, gene upregulation, gene downregulation, or a phenotypic change in response to an agent.

[0036] In a preferred embodiment of the invention, the cell, cell population, tissue, or whole organism is diseased or is derived from a diseased source. The diseased cell population, tissue, or whole organism can be diseased with any disease, malady, infection, and the like. Preferably, the disease is cancer, diabetes, an autoimmune disease, osteoporosis, or lung disease. Alternatively, the diseased cell population, tissue, or whole organism is infected with a parasite, virus, or bacteria. The diseased cell population, tissue, or whole organism can also be wounded, burned, scarred, or in a state of healing. The diseased cell also can be a protozoan, a nematode, *T. cruzi*, and leishmania.

[0037] The precise techniques for detecting the assayed activity will depend on the activity itself. For instance, if the activity is cell growth, then detection of the activity may simply comprise a cell count using a hemocytometer, visual inspection, or radioactive isotope uptake. If the activity is the production of a color-tagged product, then detection may involve detection of the color via ultraviolet-visible (UV-VIS) spectroscopy. It is well within the ordinary skill for one to determine suitable techniques for detecting the assayed activity.

[0038] Once the activity is detected, at least one entity-ligand-support complex having the entity, which exhibited the detected activity, is selected. The term "selecting" and words stemming therefrom as used herein refers to the identification of the entity-ligand-support complex, or the pool within which it resides. In the latter case, the assay may be repeated with just the constituents of the selected pool until a single entity-ligand-support complex is selected.

[0039] An advantage of the inventive methods is the ability to identify and/or characterize active entities on the basis of biological, physical, biochemical, or chemical

activity, without prior knowledge of the entity's molecular identity. Accordingly, the entity can display a biological activity and need not undergo processing (e.g., heat-inactivation) prior to practicing the inventive methods. Also, there is no need to remove more abundant proteins like albumin or active entities like immunoglobulins. Likewise, the ability of an entity to affect more specific cellular functions (e.g., production of particular proteins or other cellular constituents) might be enhanced or diminished in the assay medium, thereby providing valuable characteristics of the entity. Furthermore, the entity might be a cell that proves resistant to cytotoxic agents. Thus, the invention provides methods for the identification of novel active entities or unknown active entities (i.e., proteins not identified prior to practicing the inventive method) with specific biological activities. Also, the invention provides identification of novel or unknown biological activities for known proteins.

[0040] The way in which the methods can achieve identification of the active entities can involve determining the chemical identity of the ligand(s) that bind(s) to the entity exhibiting the assayed activity. Suitable methods of determining the chemical identity of the ligand(s) are known in the art and include, for example, mass spectrometry, Edman degradation sequencing, and the like. Once ligands have been identified as having specificity for particular entities, those ligands can be resynthesized and used to capture, isolate, detect, and/or characterize entities using, for example, chromatographic separation. To this end, the inventive methods further comprise providing multiple copies of the identified ligand and attaching each copy of the identified ligand to a support, thereby obtaining multiple ligand-support complexes. The multiple-ligand support complexes are allowed to contact a composition containing multiple copies of the entity under conditions that allow the ligand-support complexes to bind to multiple copies of the entity, thereby forming multiple entity-ligand-support complexes. The entities are dissociated from the entity-ligand-support complexes and, if desired, subjected to additional rounds of screening and/or characterization. The composition containing multiple copies of the entity used to isolate and purify the entity can be the same as the mixture that was originally used to identify the entity as an active entity. For instance, if plasma was the mixture that was originally contacted with the ligand-support complexes, then the same source of plasma can be used to isolate and purify the entities in subsequent steps.

[0041] After the active entities have been isolated and purified, the method can further comprise the step of determining the chemical or physical identity of the entity or at least further characterizing the entity, such as by performing mass spectrometry of the entity. The term "characterization" and words related thereto as used herein refer to the identification of any distinctive quality or trait of a entity, and do not require that the precise

chemical identity, e.g., the molecular formula, chemical structure, conformation, nucleotide sequence or amino acid sequence, of the entity is elucidated.

[0042] Additionally, the identified ligands also can be used in diagnostic assays, to immobilize or selectively transfer entities, and as pseudo- or synthetic receptors (see, e.g., Still, *Acc. Chem. Res.*, 29: 155-163 (1996)). Additionally, the ligands themselves can be used as therapeutic agents, catalysts, and the like.

EXAMPLES

[0043] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

[0044] For convenience, the following abbreviations are used in the examples herein: DEPFMU, 6,8-difluoro-4-methylumbelliferyl; ICAT, isotope coded affinity tag; UV-VIS, ultraviolet-visible; SAP, streptavidin-alkaline phosphatase; FITC, fluorescein isothiocyanate; ATCC, American Type Culture Collection; IL-2, interleukin-2; PNPP, p-nitro phenyl phosphate; PPV, porcine parvovirus; PVDF, polyvinylidene fluoride; AP, alkaline phosphatase; and PI, propidium iodide.

[0045] Example 1

[0046] This example demonstrates the use of the inventive methods to screen a mixture for cytotoxic factors.

[0047] The ability of a cytotoxic factor to induce cell death after being fractionated onto beads was determined. In this assay, beads to which anti-mouse TNF- α antibodies (Pharmingen) were bound, were incubated with purified mouse TNF- α (Pharmingen, San Diego, CA). Control beads were not incubated with TNF- α . In addition, controls included adding soluble TNF- α to the medium and incubating beads without antibody with TNF- α to ensure that there was no carryover of unbound TNF- α due to poor washing. The effect of plain beads was also measured. The indicated number of cells were plated per well in 24 well plates, and approximately 100 beads were added to each well of WEHI 164 cells (ATCC). After incubation with the beads for 48 hours, the indicated % cell death was observed by hemocytometer counting and trypan blue exclusion to indicate the number of dead cells (Table 1).

[0048] Table 1. Cytotoxicity Assay of TNF- α beads

	Starting Cell No.	% Dead
Soluble TNF- α	100,000	98
Anti-TNF α beads alone	100,000	0.5
Sham incubation	100,000	0.3
Plain beads	100,000	4.1
	50,000	0.1
	25,000	7.7
TNF- α coupled beads	100,000	9.1
	50,000	29.4
	25,000	12.5

[0049] This example demonstrated that sufficient amounts of protein eluted from these high-affinity ligands and caused detectable cell death in cultures of susceptible cells. These data support the thesis that active factors can be detected in multiple assays for biological function with defined endpoints.

[0050] Example 2

[0051] This example demonstrates the use of the inventive methods to screen cytokine-spiked plasma for factors that support cell growth.

[0052] The cells used in this assay were NK-92 cells (obtained from ATCC, Manassas, VA). The NK-92 cell line is a human cytotoxic T-cell line, which requires exogenous Interleukin-2 (IL-2) in order to grow. Normal cell culture medium containing serum or healthy human plasma does not have IL-2 to support NK-92 growth. Therefore, NK-92 cells normally are maintained in culture in medium supplemented with 5 ng/ml recombinant mouse IL-2.

[0053] As a model for beads bearing an IL-2 specific ligand, 300 μ g of rat anti-human IL-2 antibody (Pharmingen, San Diego, CA) was cross-linked onto 400 μ l of Protein G sepharose beads (Pierce, Rockford, IL). The beads were incubated overnight with a mixture of plasma into which a natural, secreted cytokine mixture was spiked. This mixture contained several cytokines and was derived from human monocytes and leukocytes that had been induced to secrete cytokines with phytohemagglutinin and ciprofloxacin. Media from these induced cells was subsequently pooled. The concentration of IL-2 in the spiked plasma was approximately 4 ng/ml. The beads were washed with ~50 column volumes of PBS + 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO). Beads that were incubated with 4

ng/ml pure recombinant human IL-2 were also included. Controls were beads bearing the antibody that were not incubated with IL-2 as a negative control and cultures to which soluble IL-2 was added as a positive control.

[0054] NK-92 cells were plated at 1×10^5 cells per well in 24-well plates and allowed to grow in media without added IL-2 for 24 hours to deplete the intrinsic IL-2 stores. Approximately 100 beads were added to each well. The cultures were allowed to grow for 96 hours. Cells were collected from each culture and the cell number determined by counting live cells in a hemocytometer, excluding dead cells by trypan blue exclusion. Wells in which IL-2 was provided to the cells from ligands, either as recombinant protein or purified from the secreted mixture, showed an 8- to 12-fold increase in cell number compared with the negative control. Thus, these data demonstrate that a protein was fractionated from a highly complex mixture on a high-affinity ligand, the protein diffused from the ligand at a sufficient rate and concentration into media, and its presence was detected by its ability to support the growth of dependent cells.

[0055] These results indicate that sufficient protein diffused from a high affinity ligand and its activity was detected in a relevant cell-based assay. These results can be extrapolated to predict that an unknown protein that supports growth could be identified through its activity on cells in culture and the ligand that binds that protein identified, produced in bulk, and the active protein purified by affinity chromatography, identified, and characterized.

[0056] Example 3

[0057] This example demonstrates the use of the inventive methods to identify growth factors.

[0058] Complex cytokine mixtures have been screened for proteins that support the survival of NK-92 cells using a library of hexamer peptide ligands. A natural, secreted cytokine mixture derived from isolated lymphocytes and monocytes (ImmunoRx, Inc, Farmington, NY) was used as a starting material. This mixture contained many cytokines that are released in response to biological induction and are not present in normal sera or culture media. The endpoints of this assay are both biological and fluorescent as described in Example 2. 11,000 beads from a hexamer library synthesized via a cysteine derivative on a backbone of ToyoPearl 605-M epoxy beads (synthesized by Peptides International, Louisville, KY) were incubated with the cytokine mixture. The unbound and weakly bound proteins were removed by washing with PBS (150 mM NaCl, pH 7.4). 20 – 50 beads were incubated with 40 μ l of NK-92 cells that had been plated at 2.4×10^4 cells/ml in a well of a 384-well microtiter plate. The plates were maintained at 37°C for 48 hrs. Approximately

30 clumps of growing cells were observed in 20 wells. In some cases a large clump of cells grew in close association with a bead. There were several beads in the immediate vicinity which had no cells growing near them, and significant patches of dead cells were indicated by propidium iodide (PI) uptake. One of these beads (and a few others from similar wells that supported growth) was collected and the presence of IL-2 on the beads was confirmed by modified antibody detection in a "bead blot" assay. Briefly, the beads are arrayed in agarose, the proteins transferred off the beads onto a PVDF membrane by capillary transfer in elution buffer, and the membrane probed with anti-IL-2 antibodies to detect IL-2. Eleven beads, including three potential positives were recovered, cleaned, re-incubated with the identical starting material and cultured individually with the same cell line. Two of three potential positive beads reconfirmed their activity in the deconvolution assay. An additional bead was sequenced and the associated ligand identified as the sequence GVASED (SEQ ID NO: 9). A resin with this ligand was synthesized and found to bind IL-2. The complete starting material will be fractionated on the resin and the bound proteins will be analyzed to identify additional proteins that may be purified on the resin and which contribute to the activity.

[0059] Several aspects of this type of functional screening experiment can be extrapolated to other screens for biological activity. Although IL-2 is known to be present in the starting material there are also numerous other cytokines present at various levels on the beads and in the assay that may be contributing to cell survival. NK-92 cells have been tested for their lack of responsiveness to IL-1 and several other known cytokines; however, they may be responsive to other, as yet undiscovered cytokines that are present in the starting material. Most importantly, screening for factors that are protective against cytotoxic agents and poisons can clearly be accomplished in this type of assay in which cell growth and PI exclusion are endpoints.

[0060] Example 4

[0061] This example demonstrates the use of the inventive methods to screen spiked plasma for alkaline phosphatase enzyme activity.

[0062] Streptavidin-alkaline phosphatase (500 ng) (SAP-Sigma-Aldrich) was spiked into 1 ml of pooled human plasma. The spiked plasma was incubated with ToyoPearl AF-Amino-650M beads (Tosoh BioSciences, Montgomeryville, PA) either with or without the ligand HPQFLS (SEQ ID NO: 1) (synthesized at Peptides International, Louisville, KY), a sequence known to bind streptavidin. The beads were allowed to incubate with spiked or unspiked plasma for 1 hr at room temperature, after which they were washed with HEPES buffer with 0.1% Tween-20, pH 7.2. Ligand-bearing beads were also incubated with saline

alone as a control. The beads were distributed into wells of a 384-well plate with 6 HPQFLS beads per well and 120 μ l of HEPES buffer containing 250 mM NaCl and 0.05% Tween-20 buffer. The beads were allowed to incubate for 30 hours at room temperature.

[0063] The liquid was removed from the wells and tested for the presence of alkaline phosphatase activity with p-nitro phenyl phosphate (PNPP) (Sigma-Aldrich), a colored phosphatase substrate. Yellow color developed only in those wells with buffer from ligand beads that had been exposed to AP-spiked plasma; no color was observed in the control wells. To confirm that protein did, in fact, elute from the beads, a FITC-labeled streptavidin (Pierce) was also incubated with the HPQFLS (SEQ ID NO: 1) beads as described above. The beads were then incubated with 120 μ l of 20 mM citrate 140 mM NaCl buffer for 21 hours. The buffer was collected, and fluorescence was measured in a fluorometer. The increase over baseline was 11.6%. After an additional 3 days of elution, the fluorescence increased 22.7% over baseline.

[0064] These results demonstrate that a protein with enzymatic activity was fractionated onto a high-affinity ligand and eluted from the ligand, and that the presence of the protein in solution was measurable based on its biochemical activity.

[0065] Example 5

[0066] This example demonstrates the use of the inventive methods to screen plasma for melanoma specific antibodies.

[0067] The cell line used in this assay was the human, malignant melanoma cell line SK-MEL 28. The combinatorial library was synthesized on ToyoPearl 650-M epoxy resin from Tosoh BioScience. This library was designed to have the ligands linked to the resin through the sulfhydryl group of a cysteine derivative. The starting material used as a source of IgG, IgA and IgM antibodies was a plasma fraction I+II+III paste that had been further fractionated to remove fibrinogen. The beads were incubated with paste and washed, and between 50 –150 beads were incubated with 40 μ l SK-MEL28 cells that had been plated at 1×10^4 cells/ml in wells of a 384-well plate. The media contained propidium iodide (PI), a red-fluorescent dye that is taken up only by dead cells. Images of each well were taken at 1 hour intervals for 160 hours with single cell resolution. The images were analyzed for uptake of PI over the time period, and wells in which a cumulative increase in PI uptake that was 2 standard deviations above the mean of the population were selected as being of particular interest. The beads in these wells were harvested, cleaned, re-loaded with a fresh aliquot of the original starting material, and incubated with 1-2 beads per well with the same concentration of cells and imaged in the same way as the original assay. Beads in wells in

which an increase in death above 2 standard deviations above the mean of the population was seen were collected and the associated ligand sequenced.

[0068] An increase in death in a well with a bead or in the vicinity of a specific bead indicated that a cytotoxic antibody was bound to the ligand. Any positive wells were deconvoluted by diluting the beads in the well to a single bead per well and repeating the assay. This confirmed the result and indicated the specific bead that bound the protein, whose associated ligand was sequenced. If a single bead was identified in a well without deconvolution, that bead was collected and the associated ligand sequenced.

[0069] Example 6

[0070] This example demonstrates the use of the inventive methods to screen a mixture of antibodies for antibodies of an immunized mammal.

[0071] Human plasma-derived intravenous immunoglobulin (IgG) was manufactured from pools of up to 60,000 donors and included vast varieties of antibodies with diverse affinities, some of which bind to cell surface receptors. Populations of antibodies can also be raised that are directed against cell surface receptors by immunizing mice with membrane preparations. The antibodies can be used as screening starting materials to identify their receptor epitopes in methods such as the "bead blot" receptor-binding antibodies may be useful for the upregulation of PON1. Specific epitopes for antibodies in a differential screen were identified using purified IgG preparations from normal and immunized mice. One cohort of mice was immunized with ovalbumin; unimmunized mice were used as controls. IgG preparations from each group were purified from pooled sera using affinity chromatography on protein G sepharose (Pierce), and the two populations were differentially labeled with either Alexa 488 (green) or Alexa 568 (red) dye (Molecular Probes, Eugene OR). The two IgG populations were then mixed and incubated with a library of combinatorial hexamer ligands. The beads were washed extensively and observed under a fluorescence microscope. The majority of beads that fluoresced had both red and green signals, indicating that they bound antibodies that were present in both populations. Beads that fluoresced only red were indicative of antibodies that were present only in the immunized population. These were collected and sequenced. Several showed very strong sequence similarity to ovalbumin, e.g. ILRVIR (SEQ ID NO: 2) has homology with the ovalbumin sequence RTINKVVRF (SEQ ID NO: 3), IFDKVQG (SEQ ID NO: 4) homology with RFDKLPFG (SEQ ID NO: 5) and PPFRIHG (SEQ ID NO: 6) homology with MPFRVITE (SEQ ID NO: 7). Several of the remaining decoded sequences had high homology with bacterial sequences that are believed to have arisen from the Freund's adjuvant used during immunization.

[0072] Example 7

[0073] This example demonstrates the use of the inventive methods to identify enzymes bound to libraries by measuring paraoxonase activity.

[0074] A ligand that binds HDL was screened for its ability to purify paraoxonase from plasma by measuring the enzyme's activity. Paraoxonase is implicated in protecting against the build up of atherosclerotic plaque, as well as detoxification of some nerve poisons (sarin) and insecticides. 5 µg of the ligand 2'-naphthyl-alanineWLHAN (SEQ ID NO: 8) was incubated with 100 µl of 1:10 diluted rabbit serum in PBS for one hour at 37° C. The beads were centrifuged to remove the supernatant and the supernatant stored in eppendorf tubes. The resin bead pellets were resuspended in an equal volume of paraoxonase assay buffer. Paraoxonase activity of the supernatants was measured using a sensitive, specific, fluorescent substrate. 100 µM DEPFMU (6, 8-difluoro-4-methylumbelliferyl) was mixed with 10 µl supernatant, 10 µl of bead suspension, or 10 µl serum in a standard microtiter plate for 20 minutes at 37° C. Hydrolysis of DEPFMU was quantified by measuring fluoresce at 355 nm emission and 460 nm excitation using a commercial fluorometer. Hydrolysis was quantified compared with a standard curve of DEPFMU activity. 30% of the starting activity was detected in wells with the resins, whereas less than 4 % of the original paraoxonase activity remained in the serum. These data indicate that ligands that bind an enzyme can be detected through that enzyme's activity associated with a bead, and that enzymes themselves can be discovered from complex mixtures using this assay to screen for activity.

[0075] Example 8

[0076] This example demonstrates the use of the inventive methods to screen libraries for ligands that bind virus based on screening for virus-associated cytotoxicity.

[0077] Ligands that bind porcine parvovirus (PPV) were identified using the inventive methods. This assay was a modification of the classical plaque assay, in which infectivity is measured by viral plaques formed by lysis of susceptible mammalian cells in culture. As a model for ligands that bind virus, 2 µl of polyclonal porcine anti-PPV antibody was conjugated to Protein A sepharose beads according to the manufacturer's protocol (Pierce). The beads were incubated with 8 logs of PPV purified from PK13 cells by serial centrifugation. These beads were mixed with low-melting point agarose and spread over the surface of a 70% confluent culture of PK 13 cells. Following five days incubation at 37°C/ 5% CO₂, the live cells were stained with Neutral Red stain. There were evident areas of cell

death surrounding beads that had bound virus. These results demonstrate that the controlled diffusion of active entities (viruses) from beads indicated the active bead.

[0078] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0079] The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0080] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

WHAT IS CLAIMED IS:

1. A method of screening a mixture for active entities, which method comprises:
 - (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes,
 - (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex,
 - (iii) separating at least one entity-ligand-support complex from the unbound entities,
 - (iv) assaying at least one entity of at least one separated entity-ligand-support complex for an activity,
 - (v) detecting the activity, and
 - (vi) selecting at least one entity-ligand-support-complex having the entity, which exhibited the detected activity,whereupon a mixture is screened for active entities.
2. The method of claim 1, wherein the ligands are selected from the group consisting of cells, bacteria, viruses, yeast, proteins, peptides, amino acids, nucleic acids, carbohydrates, lipids, drugs, synthetic inorganic compounds, synthetic organic compounds, isoforms of any of the foregoing, and combinations of any of the foregoing.
3. The method of claim 2, wherein the ligands are peptides, and the peptides are generated by combinatorial approaches.
4. The method of claim 1, wherein the support comprises a material selected from the group consisting of polymethacrylates, polyacrylates, agarose, polyacrylamides, dextran, cellulose, polysaccharides, nitrocellulose, silicon, styrene, polyethylene-coated polystyrene, metal, polyvinylidifluoride, nylon, and combinations of any of the foregoing.
5. The method of claim 1, wherein the mixture is a biological fluid, an environmental extract, or a composition comprising chemical compounds.
6. The method of claim 5, wherein the biological fluid is selected from the group consisting of blood, plasma, pooled plasma, intermediates from plasma fractionation, serum,

a cell homogenate, a tissue homogenate, a conditioned medium, a fermentation broth, cerebrospinal fluid, urine, saliva, milk, ductal fluid, tears, perspiration, lymph, semen, umbilical cord fluid, and amniotic fluid.

7. The method of claim 5, wherein the biological fluid is a plasma-derived fraction comprising antibodies and anti-idiotypic antibodies.

8. The method of claim 5, wherein the biological fluid is obtained from a host afflicted with a disease.

9. The method of claim 5, wherein the environmental extract is selected from the group consisting of a soil extract, an extract from a naturally-occurring body of water, a sample of ice, air, ash, rock, or permafrost, and a swab from a building.

10. The method of claim 5, wherein the composition comprising chemical compounds comprises natural or synthetic chemical compounds.

11. The method of claim 1, wherein the entities are selected from the group consisting of proteins, peptides, drugs, antibodies, cells, synthetic molecules, organic compounds, protein complexes, bacteria, viruses, and fungi.

12. The method of claim 1, wherein the activity is a biological, physical, chemical, or biochemical activity.

13. The method of claim 12, wherein the activity is an enzyme activity or inhibition of an enzyme activity.

14. The method of claim 12, wherein the activity is an effect on a cell, a cell population, a tissue, or a whole organism.

15. The method of claim 14, wherein the activity is an effect on a cell or a cell population and the effect is selected from the group consisting of cell migration, cell proliferation, cell death, cell differentiation, cell cycle entry, cell cycle arrest, apoptosis, cell lysis, growth arrest, cell survival, a change in an intracellular signaling pathway, antigen expression, gene upregulation, gene downregulation, and a phenotypic change in response to an agent.

16. The method of claim 14, wherein the cell, cell population, tissue, or whole organism is diseased.
17. The method of claim 16, wherein the diseased cell, cell population, tissue, or whole organism is diseased with cancer, diabetes, an autoimmune disease, osteoporosis, or lung disease, infected with a parasite, virus, or bacteria, wounded, burned, scarred, or in a state of healing.
18. The method of claim 1, wherein multiple entity-ligand-support complexes are formed in step (ii).
19. The method of claim 1, which further comprises, after step (iii) and before step (iv), a sub-pooling step, wherein at least one separated entity-ligand-support complex and the ligand-support complexes are separated into several pools.
20. The method of claim 1, which further comprises (vii) determining the chemical identity of at least one ligand to which at least one entity exhibiting the detected activity binds.
21. The method of claim 20, which further comprises
- (viii) providing multiple copies of at least one ligand identified in step (vii),
 - (ix) attaching each copy to a support, thereby obtaining multiple ligand-support complexes,
 - (x) contacting the ligand-support complexes with a composition comprising multiple copies of at least one entity exhibiting the detected activity under conditions that allow the ligand-support complexes to bind to multiple copies of at least one entity exhibiting the detected activity, thereby forming multiple entity-ligand-support complexes, and
 - (xi) dissociating the entities from the entity-ligand-support complexes, thereby recovering the entities from the composition.
22. The method of claim 21, wherein the composition of step (x) is the same as the mixture of step (ii).
23. The method of claim 21, which further comprises

(xii) determining the chemical or physical identity of the entity exhibiting the detected activity.

24. The method of claim 19, which further comprises, after the sub-pooling step and before step (iv), an eluting step, wherein the entities of the multiple entity-ligand-support complexes are dissociated from the complexes, and the ligand-support complexes are subsequently removed from the pools.

25. The method of claim 19, which method comprises, after the sub-pooling step and before step (iv), a step, wherein a semi-solid or viscous material is added to each pool, wherein the entity of at least one entity-ligand-support complex dissociates from the complex and diffuses into the material, thereby forming a concentration gradient of the entity, wherein the concentration of the entity gradually decreases as the distance from the ligand-support complex from which the entity dissociated increases.

26. A method of screening a mixture for active entities, which method comprises:

- (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes,
 - (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex,
 - (iii) separating at least one entity-ligand-support complex and the ligand-support complexes from the unbound entities,
 - (iv) separating at least one entity-ligand-support complex and the ligand-support complexes into pools,
 - (v) dissociating at least one entity from at least one separated entity-ligand-support complex,
 - (vi) removing from the pools the ligand-support complexes or the at least one dissociated entity of step (v),
 - (vii) assaying at least one dissociated entity of step (v) for an activity,
 - (viii) detecting the activity, and
 - (ix) selecting at least one entity exhibiting the detected activity,
- whereupon a mixture is screened for active entities.

27. A method of screening a mixture for active entities, which method comprises:

- (i) providing a plurality of ligands, wherein each ligand is attached to a support to form a plurality of ligand-support complexes,
 - (ii) contacting the ligand-support complexes with a mixture comprising a plurality of entities under conditions that allow at least one entity to bind to at least one ligand-support complex, thereby forming at least one entity-ligand-support complex,
 - (iii) separating at least one entity-ligand-support complex and the ligand-support complexes from the unbound entities,
 - (iv) separating at least one entity-ligand-support complex and the ligand-support complexes into pools,
 - (v) adding a semi-solid or viscous material to each pool, wherein the entity of the at least one separated entity-ligand-support complex dissociates from the complex and diffuses into the material, thereby forming a concentration gradient of the entity, wherein the concentration of the entity gradually decreases as the distance from the ligand-support complex from which the entity dissociated increases,
 - (vi) assaying at least one dissociated entity of step (v) for an activity,
 - (vii) detecting the activity, and
 - (viii) selecting at least one entity exhibiting the detected activity,
- whereupon a mixture is screened for active entities.

SEQUENCE LISTING

<110> The American National Red Cross

<120> METHOD FOR IDENTIFYING INDIVIDUAL ACTIVE ENTITIES FROM COMPLEX MIXTURES

<130> 222362

<150> US 60/395,038

<151> 2002-07-11

<160> 9

<170> PatentIn version 3.2

<210> 1

<211> 6

<212> PRT

<213> Artificial

<220>

<223> Synthetic

<400> 1

His Pro Gln Phe Leu Ser
1 5

<210> 2

<211> 6

<212> PRT

<213> Artificial

<220>

<223> Synthetic

<400> 2

Ile Leu Arg Val Ile Arg
1 5

<210> 3

<211> 9

<212> PRT

<213> Artificial

<220>

<223> Synthetic

<400> 3

Arg Thr Ile Asn Lys Val Val Arg Phe
1 5

<210> 4

<211> 7

<212> PRT

<213> Artificial

<220>

<223> Synthetic

<400> 4

Ile Phe Asp Lys Val Gln Gly

1 5

<210> 5
<211> 9
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 5

Arg Phe Asp Lys Leu Pro Gly Phe Gly
1 5

<210> 6
<211> 7
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 6

Pro Pro Phe Arg Ile His Gly
1 5

<210> 7
<211> 8
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 7

Met Pro Phe Arg Val Ile Thr Glu
1 5

<210> 8
<211> 5
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 8

Trp Leu His Ala Asn
1 5

<210> 9
<211> 6
<212> PRT
<213> Artificial

<220>
<223> Synthetic

<400> 9

Gly val Ala Ser Glu Asp
1 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/19584

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/00, 1/68, 1/70; G01N 33/53, 33/566, 33/567

US CL : 435/ 4-6, 7.1, 7.21; 436/ 501, 503, 504, 808

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 4-6, 7.1, 7.21; 436/ 501, 503, 504, 808

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/40265 A2 (V.I. TECHNOLOGIES, INC.) 07 June 2001, see entire document.	1-24 & 26
X	US 5,482,867 A (BARRETT et al.) 09 January 1996, see entire patent.	1-18 & 20
X	WO 92/00091 A1 (BIOLIGAND, INC.) 09 January 1992, see entire document.	1-27

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

21 August 2003 (21.08.2003)

Date of mailing of the international search report

20 OCT 2003

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US

Commissioner for Patents

P.O. Box 1450

Alexandria, Virginia 22313-1450

Facsimile No. (703)305-3230

Authorized officer

Bennett Celsa

Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US03/19584

Continuation of B. FIELDS SEARCHED Item 3:

WEST: USPT, JPAB, EPAB, DWPI

STN: CAPLUS, EMBASE, BIOSIS, MEDLINE, WPIDS

Form PCT/ISA/210 (second sheet) (July 1998)

